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Metabolic memories

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CHAPTER 2

Oxidative stress *in utero* protects against diet-induced obesity and insulin resistance in adult male *Ldlr*-receptor knockout mice

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ABSTRACT

Background: Pregnancy complications such as preeclampsia and hypertension are a frequent cause of fetal growth restriction and increased fetal oxidative stress, and associate with a higher incidence of the metabolic syndrome in adulthood. However, the pathophysiological contribution of oxidative stress per se is experimentally difficult to address and has not been investigated. The present study aimed to determine the effects of increased intrauterine oxidative stress on adiposity, glucose and cholesterol metabolism in adult offspring in a preclinical model.

Methods: We crossed either male *Ldlr*^{-/-}*xSod2*^{+/+} mice with *Ldlr*-knockout dams heterozygous for *Sod2* (+/-, increased intrauterine oxidative stress, IUOx) or male *Ldlr*-knockout heterozygous for *Sod2* (+/-) with *Ldlr*^{-/-}*xSod2*^{+/+} dams (control) and followed *Sod2*^{+/+} offspring into adulthood. At 12 weeks of age, the mice received Western diet for an additional 12 weeks followed by characterization of metabolic parameters until 24 weeks of age.

Results: Despite equal embryonic weight, adult male IUOx offspring displayed lower body weight (-12%, $p < 0.05$) and reduced adiposity (-23%, $p < 0.05$) compared to controls. This was associated with improved glucose tolerance both before (12 weeks) and after (24 weeks) the dietary challenge ($p < 0.05$). Conceivably, reduced weight gain in IUOx was due to increased energy dissipation in white adipose tissue conveyed by a 3-fold increased expression of Ucp1 ($p = 0.05$). Female offspring did not display a comparable phenotype.

Conclusions: The results of this study demonstrate protective effects of isolated fetal oxidative stress against the obesogenic effects of Western diet in adulthood. Our data further indicate that early life exposure to reactive oxygen species programs energy dissipation in white adipose tissue at the level of Ucp1.

INTRODUCTION

Metabolic syndrome (MetS) is a modern lifestyle pandemic that comprises a number of pathophysiologies defined by the development of obesity, insulin resistance, type 2 diabetes, and dyslipidemia³⁰⁸. The causes for the recent steep increase in the incidence of MetS seem to be rooted not only in the interplay of an adverse environment and predisposing genetics but also in environmental effects in the early stages of life when epigenetic imprinting occurs³⁰⁹. Increasing epidemiological evidence indicates that stressful environmental conditions during sensitive periods of early development are linked to a predisposition to chronic disease later in life^{310,311}. Intrauterine growth restriction (IUGR) is one particular manifestation of fetal stress which has been associated with an increased visceral obesity³¹² in adulthood and a higher incidence of hypertension and ischemic heart disease³. While it can be induced by nutritional insufficiency as demonstrated by uterine artery ligation in animal models³¹³, IUGR is also one of the consequences of preeclampsia³¹⁴ and maternal smoking³¹⁵, which have been implicated to strongly impact the developing offspring with life-long negative metabolic consequences³¹⁶⁻³¹⁸.

Interestingly, a common denominator of these adverse perinatal conditions is fetal oxidative stress³¹⁹. Oxidative stress can be a mere accompanying feature of an increased metabolic burden. Alternatively, it could represent the underlying driving force for metabolic programming in these cases, since it was shown that increased levels of reactive oxygen species (ROS) mechanistically contribute to atherosclerotic plaque formation³²⁰ and pancreatic β -cell dysfunction⁵⁸. However, so far the involvement of oxidative stress in the predisposition towards components of MetS and the role it plays in the etymology of fetal metabolic programming has not been investigated. What makes defining the role of oxidative stress challenging is the fact that it co-manifests with other programming conditions, such as IUGR. In such scenarios the respective effects of different programming stimuli could be masking each other, thereby rendering conclusions on causality difficult.

Here, we aimed to address this issue by investigating the specific impact of isolated intrauterine oxidative stress (IUOx) on obesity and related components of the metabolic syndrome in adulthood. Therefore, we used a genetic mouse model where intrauterine oxidative stress is not associated with either IUGR, maternal obesity, diabetes or dyslipidemia. Specifically, we compared *Sod2*^{+/+} offspring derived from matings of *Sod2*^{+/-} mothers with *Sod2*^{+/+} fathers (high intrauterine oxidative stress) to *Sod2*^{+/+} offspring from *Sod2*^{+/+} mothers with *Sod2*^{+/-} fathers (controls). With this approach, we were able to demonstrate that isolated intrauterine oxidative stress protects against the development of diet-induced obesity in male adult offspring. Conceivably, the protective effect is conveyed by programming of energy dissipation in white adipose tissue via increased expression of Ucp1.

MATERIALS AND METHODS

Animal experiments

We aimed to generate a preclinical model, in which offspring are exposed to high-intrauterine levels of ROS without confounding by the offspring's own genotype or fetal growth restriction. To address this aim, mice sharing the same genetic background, heterozygous for *Sod2*^{tm1Leb} and homozygous for *Ldlr*^{tm1Her} were purchased from Jackson Laboratories (stock #006883, Bar Harbor, ME, USA) and crossed with *Ldlr*-knockout mice (stock #002207). Thus, all respective offspring are homozygous *Ldlr*-knockouts (-/-). *Ldlr*-knockout offspring, wild-type for the *Sod2*-mutation, which originated from females heterozygous for *Sod2* (+/-) mated with *Ldlr*-knockout males (*Sod2*+/+), were designated **IUOx**. These were compared with offspring with the same genotype (*Ldlr*-/- x *Sod2*+/+) but originating from *Ldlr*-knockout females (*Sod2*+/+) mated with *Sod2*+/+ x *Ldlr*-/- males (designated **Control**). The littermates heterozygous for the *Sod2*-mutation were not considered in this study due to potential overriding effects of increased oxidative stress caused by their own genotype. At embryonic day E18.5, we collected fetuses to measure fetal body weight for model validation. Additional pups were weaned and followed into adulthood for symptoms of obesity and metabolic syndrome development in conditions of prolonged Western-type diet feeding. The animals were co-housed in quiet, temperature-controlled conditions with 12:12 hours light/dark cycle. All breeding was performed on AIN93G semisynthetic diet (#D10012G, Research Diets, New Brunswick, NJ, USA), which was provided ad libitum also to the weaned offspring until 12 weeks of age after which the experimental animals received Western diet containing 60% fat and 0.25% cholesterol (Research Diets, #D14010701). The experimental protocol was approved by the IACUC at the University of Groningen (protocol DEC6493AA). Animal experiments adhered to the ARRIVE standards. A time line summarizing the detailed set-up of the study is shown in **Figure 1**.

Body composition analysis

After 12 weeks of Western diet body composition analysis was performed by dual energy x-ray absorptiometry (pDEXA, Norland-Stratec, Norland Medical Systems Inc., Basingstoke, Hampshire, UK). During the procedure the animals were anesthetized with isoflurane for a total of 15 minutes. Fat and lean body mass were calculated based on the automated bone mass density evaluation.

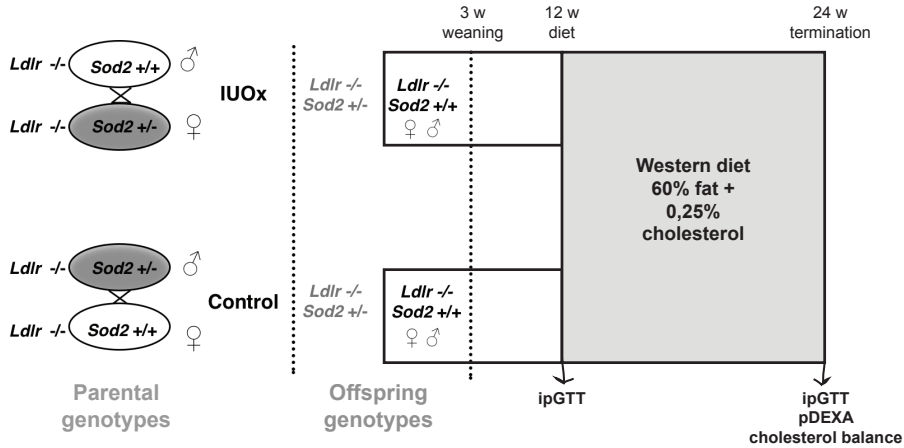


Figure 1: Schematic representation of the experimental design. The background of all mice was *Ldlr*- knockout. To obtain a model for isolated intrauterine oxidative stress (IUOx) *Sod2*^{+/-} dams were crossed with *Sod2*-wild-type males. *Sod2*^{+/-} IUOx offspring were then compared with *Sod2*^{+/-} offspring from crosses of *Sod2*^{+/-} females with *Sod2*^{+/-} males

(Control). Littermates heterozygous for the *Sod2*^{tm1Leb} mutation were not considered in this study due to the overriding effects of increased oxidative stress caused by their own genotype. At 12 weeks of age all offspring were allowed ad-libitum Western diet containing 60% fat and 0.25% cholesterol. Before and after provision of the diet glucose tolerance testing was performed. Following 12 weeks on Western diet we measured body composition via pDEXA and characterized cholesterol metabolism.

Fat balance

The fat absorption efficiency was determined based on the balance between dietary intake of long chain fatty acids and their amount recovered from feces. The fatty acids were methylated and extracted from food and fecal samples as described³²¹ followed by determination by gas chromatography. Briefly, 50 mg of material was dissolved in 10 mL CHCl₃/MeOH (2:1 volume ratio) and transferred to glass tubes with teflon liner caps (Kimax, Kimble-Chase, Vineland, NJ, USA.). Methylation was done with Methanol/HCl (6N) (5:1 volume ratio) in the presence of dibutylhydroxytoluene for 4 hours at 90 °C. The methylated fatty acids were extracted via two rounds of hexane extraction and measured with gas chromatography. The free fatty acid concentration of the species 14:0 to 26:0 per gram of sample was calculated relative to a 17:0-internal standard.

Glucose tolerance test and insulin measurement

At 12 and 24 weeks of age glucose tolerance tests were performed following a 12-hours overnight fast. The mice were injected i.p. with 1.25 g/kg D-glucose and plasma glucose was measured before and 15, 30, 60 and 120 minutes after the injection using OneTouch Ultra glucose strips (Life Scan, Milpitas, CA, USA). Circulating insulin levels

were determined using the Ultrasensitive Insulin ELISA Kit (Chrystal Chem, Downers Grove, IL, USA). HOMA-IR was calculated as previously described³²².

Plasma lipids and lipoprotein profiles

At the end of the study, blood was collected via retro-orbital bleeding under isoflurane anesthesia. The samples were centrifuged at 500 xg, 4 °C for 10 min. Total cholesterol and triglycerides were measured with colorimetric commercially available kits (Roche, Mannheim, Germany). Pooled plasma samples (n=4-10/group) were diluted 5 times and loaded onto a Superose 6 HR 10/300 GL column (GE Healthcare Little Chalfont, UK) for lipoprotein separation via fast protein liquid chromatography (FPLC) as described³²³. Total cholesterol in the collected individual fractions was measured as detailed above.

Fecal neutral sterols and bile acids in feces

Feces were dried, weight and ground to powder. Neutral sterols and bile salt profiles were determined as previously described³²⁴. Briefly, 50 mg feces were saponified in the presence of 1 mL alkaline methanol (1:3 NaOH: methanol) by heating for 2 hours at 80 °C. The neutral sterols were extracted with petroleum ether, derivatized in a mixture of BSTFA, pyridine and TMCS in a ratio of 5:5:0.1, re-dissolved in heptane containing 10% BSTFA and measured by gas chromatography. Following neutral sterol extraction, total bile acids were extracted from the aqueous phase using SepPak® C18 cartridges (Waters, Milford, MA, USA), methylated and, following a derivatization with BSTFA, pyridine, and TMCS, measured by gas chromatography in the same manner as the neutral sterols.

Gene expression

Gene expression was analyzed by quantitative real-time PCR. RNA was isolated from frozen tissues using Trizol® reagent according to the manufacturer's instructions (Thermo Fischer Scientific, Waltham, MA, USA). Quantification was performed using a NanoDrop 2000 (Thermo Fischer Scientific). A total of 1 µg of RNA was used for cDNA synthesis with M-MLV reverse transcriptase (Sigma, St. Louis, MS, USA). Real-time PCR was carried out using the StepOne RealTime PCR system (Applied Biosystems, Foster City, CA, USA). Target gene expression levels were normalized to the expression of the housekeeping gene *36b4*.

Statistical analysis

Statistical analysis of the data was performed using Graph Pad Prism 6.0 (Graph Pad Software). Data are presented as median and interquartile range (Tukey plots) unless otherwise indicated. Differences between groups were calculated using non-parametric statistical tests (Mann-Whitney or Kruskal-Wallis followed by post hoc Tukey) or repeated measures ANOVA where appropriate followed by Fischer's exact test. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Intrauterine oxidative stress caused by maternal deficiency of *Sod2* does not affect embryonic growth

Embryonic development takes place in a predominantly hypoxic environment, in which the redox balance drives tissue differentiation and is essential for survival⁶¹. While full ablation of *Sod2* is lethal, heterozygosity causes various pathophysiological manifestations in the fetus, including growth retardation³²⁵. Our aim was to produce non-growth restricted offspring wild type for *Sod2* that are exposed to increased levels of oxidative stress only due to the maternal conditions, but not to their own genotype (IUOx). In order to validate our model for animals non-confounded by the mutation, and measure the impact of maternal oxidative stress on fetal growth, we collected 18.5 days old embryos from IUOx and control dams. Intrauterine growth restriction did not manifest in pups wild-type for the *Sod2* gene originating from *Sod* +/- dams (**Figure 2A**). This demonstrates that in our model any adult phenotype would occur independent of fetal growth restriction.

Intrauterine oxidative stress associates with reduced body weight gain and improved glucose tolerance after dietary challenge in adult male offspring

At 12 weeks of age *Sod2*-wild-type offspring were given a challenge diet containing 60% fat and 0.25% cholesterol. Although the body weight of the IUOx and control group did not differ at the start of the diet, from week 6 onwards there was a delay in the weight gain of the male IUOx mice (**Figure 2B**), which continued until the end of the experiment. This effect was observed only in the male offspring while females had comparable body weight gain (**Supplementary figure 1A**). In general, lower body weight associates with better glucose tolerance³²⁶. However, yet before the start of the Western diet challenge, at 12 weeks of age, when the body weight of the groups was still comparable, IUOx-males already showed a tendency towards improved glucose tolerance (**Figure 2C, 2E**, $p=0.083$) compared to controls in an intraperitoneal glucose tolerance test. After 12 weeks on Western diet, both groups had become more glucose intolerant ($p<0.01$, **Fig. 2E**) than prior to the challenge. The IUOx-males, however, still performed better displaying reduced plasma glucose levels during the test (**Figure 2D, 2E**, $p<0.01$). Fifteen minutes after the glucose injection plasma samples were collected for determination of circulating insulin, which was significantly lower ($p<0.05$) in IUOx-mice (**Figure 2F**). Consequently, the insulin resistance index (HOMA-IR) was lower ($p<0.05$) in the IUOx group compared to control mice (**Figure 2G**).

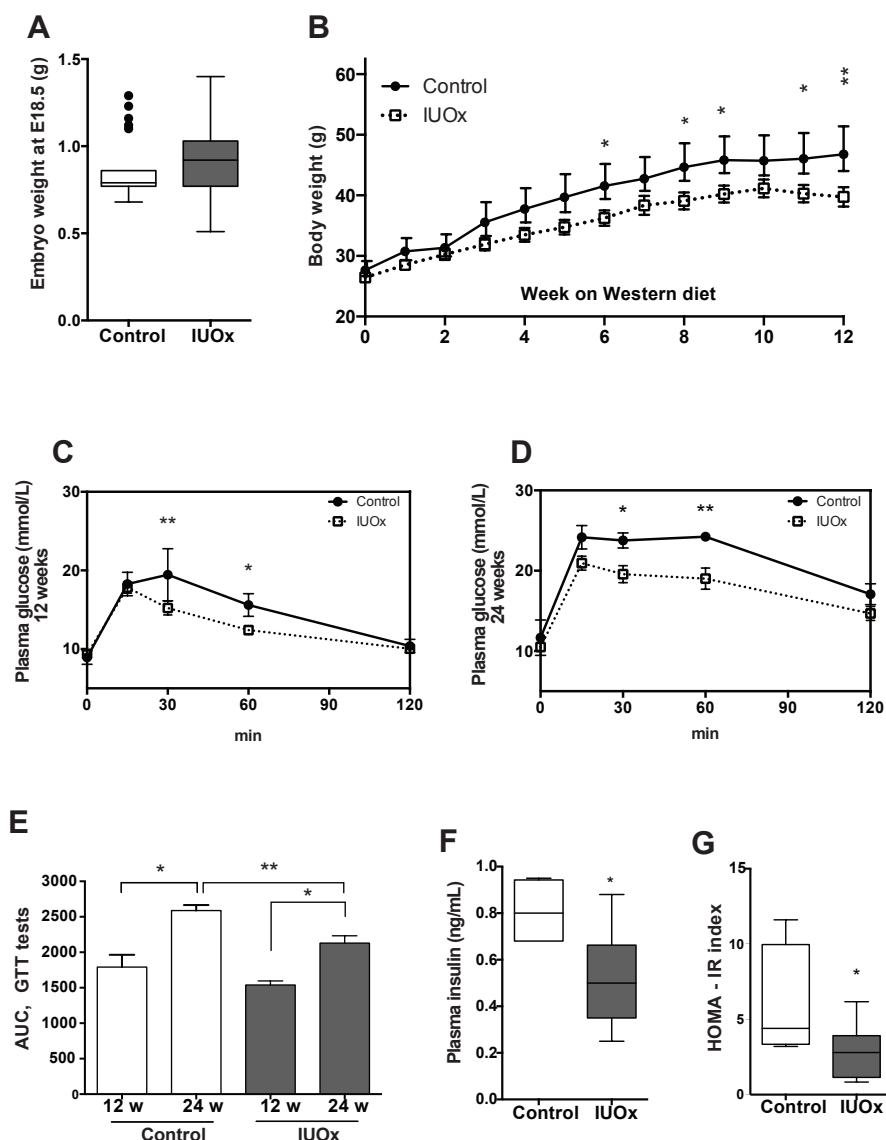


Figure 2: IUOx protects against diet-induced obesity and insulin resistance in adulthood. A) Embryonic weight at 18.5 days. Data are median and interquartile range; $n > 12$ /group; Mann-Whitney U-test. B) Body weight trajectories from the start of Western diet feeding until 24 weeks of age. C) Glucose tolerance test in 12 weeks old offspring fed chow diet. D) Glucose tolerance test at 24 weeks, after 12 weeks on Western diet. Repeated measures ANOVA posthoc Fischer's exact test. E) Calculated areas under the curve following the glucose tolerance tests at 12 and 24 weeks of age. Kruskal-Wallis H-test. F) Glucose-induced insulin secretion after 12 weeks of Western diet. G) HOMA-IR after 12 weeks on WD; reference values for fasted blood glucose and insulin were obtained from control animals prior to the diet. Data is median and interquartile range; males, $n = 4-13$ /group. Mann-Whitney U-test. * $p < 0.05$, ** $p < 0.01$.

Intrauterine oxidative stress results in lower plasma cholesterol in adult male offspring

Ldlr-knockout mice are susceptible to diet-induced hyperlipidemia³²⁷. Following 12 weeks of dietary challenge, IUOx males had a 22% reduction ($p < 0.05$) in fasted plasma total cholesterol compared to control animals (Figure 3A) accompanied by lower, though not statistically significant, plasma TG ($p = 0.075$, Figure 3B). This reduction was mostly due to lower cholesterol within Apo-B containing lipoproteins (Figure 3C). Next, we investigated fecal sterol excretion. While cholesterol recovered from feces was comparable between the groups (Figure 3E), fecal bile acid output was higher in IUOx mice (Figure 3G). Such effects on plasma lipid levels and fecal sterol excretion were absent in the female offspring (Supplementary figure I C-E).

Male IUOx offspring display reduced adiposity due to browning of white adipose tissue

To investigate potential causes for the observed difference in body weight between the groups, we performed body composition analysis before termination. We observed a marked reduction in the amount of adipose tissue present in IUOx, while no differences were detected in lean body mass (Figure 4A). Decreased adiposity could be explained by either lower caloric intake or increased energy expenditure. There was no apparent difference in the caloric intake per day (Figure 4B, left) or in the fat absorption efficiency as determined by the amount of long-chain fatty acids recovered from feces (Figure 4B, right). As a surrogate parameter for energy expenditure, we measured mRNA expression levels of genes associated with increased energy dissipation in brown and white adipose tissue. Uncoupling protein 1 (*Ucp1*) is a key mediator of increased energy expenditure³²⁸. Usually expressed in brown adipose tissue in a *Pgc1 α* -dependent manner upon norepinephrine-cAMP stimulation, its upregulation in white adipocytes in the context of so-called ‘browning’ has been shown to result in decreased obesity³²⁹. We observed a marked 3-fold induction of *Ucp1* mRNA ($p = 0.05$) in white adipose tissue of the IUOx group (Figure 4C). At the same time, there was no evident change in *Ucp1* expression in brown adipocytes. Consistent with the lack of differential weight gain in the female offspring expression levels of *Ucp1* in female mice were comparable between the control and the IUOx groups (Supplementary figure IF).

***Pgc1 α* -independent mechanisms determine increased *Ucp1* expression in adulthood following intrauterine oxidative stress**

To gain further mechanistic insights, we determined the mRNA expression of key transcriptional regulators of *Ucp1*. Its upregulation in white adipocytes has been previously described in *Ppar γ* knockout mice, which are resistant to diet-induced

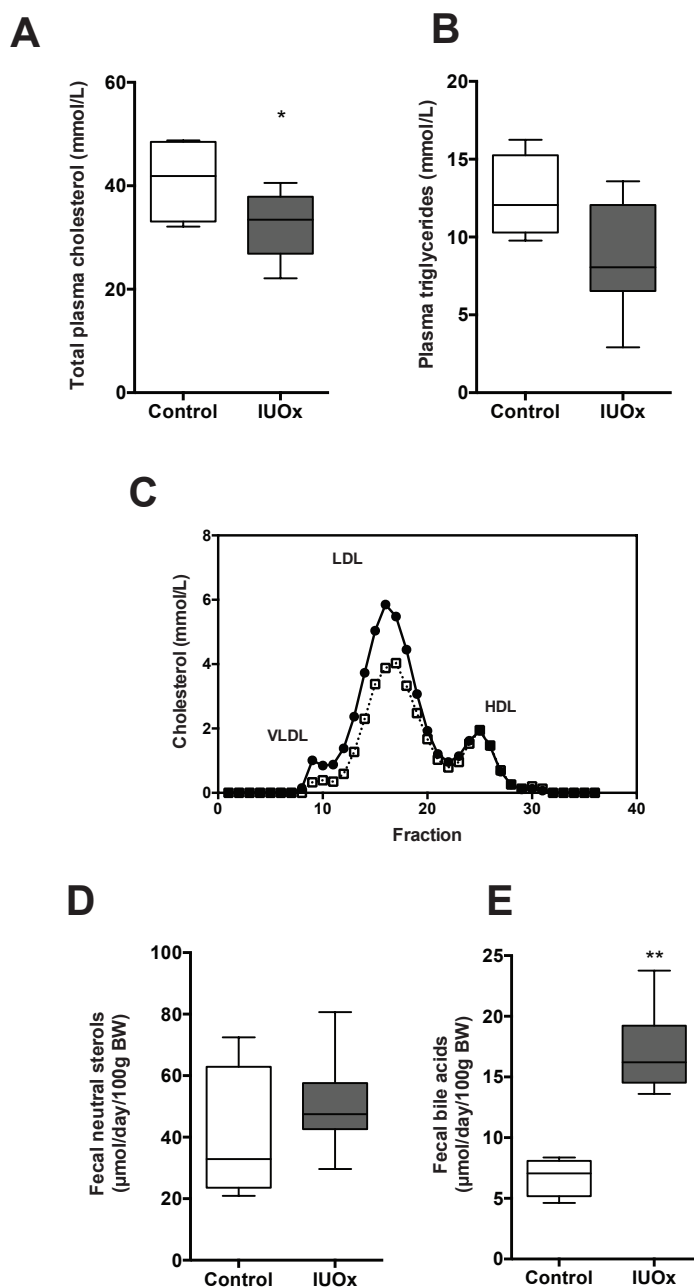


Figure 3: I/Ox protects against the effects of Western diet on cholesterol metabolism. A) Total plasma cholesterol levels at 24 weeks of age. B) Plasma triglycerides at 24 weeks. Data is median \pm range; $n=4-13$ /group, Mann-Whitney U-test. C) Cholesterol distribution over the different lipoprotein fractions following FPLC separation of pooled plasma samples of at least 4 mice/group at the age of 24 weeks. D) Fecal neutral sterol and E) total fecal bile acid excretion at 24 weeks of age. Data are presented as median and interquartile range (Tukey), $n=4-13$ / group, Mann-Whitney U-test, * $p<0.05$., ** $p<0.01$

obesity³³⁰. However, expression of both Ppar γ and its model target gene Cd36, responsible for fatty acid uptake, were comparable between IUOx and control mice in different tissue samples tested (**Figure 5A-B**), indicating that likely Ppar γ is not causally involved in the regulation of Ucp1 in our model.

The expression levels of Ucp1 can also be modulated by reactive oxygen species³³¹. We hence measured the expression of key genes involved in regulating oxidative metabolism, such as the Ppar γ co-activator 1 α , Pgc1 α ³³², which e.g. has been demonstrated to mediate norepinephrine-stimulated Ucp1-dependent adaptive thermogenesis in brown adipose tissue²⁶⁷. Importantly, Pgc1 α has been assigned a main role in the browning of white adipose tissue³³³ and it appears to be an important mediator of oxidative stress-induced upregulation of Ucp1³³⁴. While its mRNA levels were abundant in liver and brown adipose tissue, mirroring the expression of Ppar γ , Pgc1 α was generally expressed at low levels in white adipocytes, and also no differences in expression were detected between the IUOx and the control group (**Figure 5C**). In addition, another transcriptional target of norepinephrine stimulation with relevance to the redox state of the tissue, hypoxia-inducible factor 1 alpha (Hif1 α)³³⁵, was downregulated in white adipocytes from IUOx mice (-22%, $p < 0.05$, **Figure 5D**), thereby also ruling out this pathway for potential regulation. Combined these data suggest the existence of a yet undescribed, likely epigenetic, mechanism for Ucp1 regulation in response to intrauterine oxidative stress operating independent of the classical norepinephrine/Pgc1 α -mediated activation.

DISCUSSION

The results of this study demonstrate that, independent of fetal weight, increased levels of intrauterine oxidative stress have a lasting, sexually dimorphic positive impact on obesity and metabolism in adult offspring. Our data suggest that intrauterine oxidative stress programs protection against diet-induced gain in adiposity, insulin resistance, and hyperlipidemia. This is conceivably achieved by an increase in energy expenditure and browning of white adipose tissue as indicated by increased local Ucp1 expression.

Multiple pregnancy complications, like preeclampsia or pre-conceptional malnutrition, which are accompanied by increased levels of oxidative stress in utero (IUOx) as well as intrauterine growth restriction (IUGR), have been linked to higher incidence of metabolic syndrome-related pathophysiologies in the offspring. However, due to confounding and the unavailability of a suitable model for isolated IUOx, so far there have been only speculations about the contribution of oxidative stress to the development of metabolic dysfunction in adult offspring. Increased ROS production has multiple adverse effects in

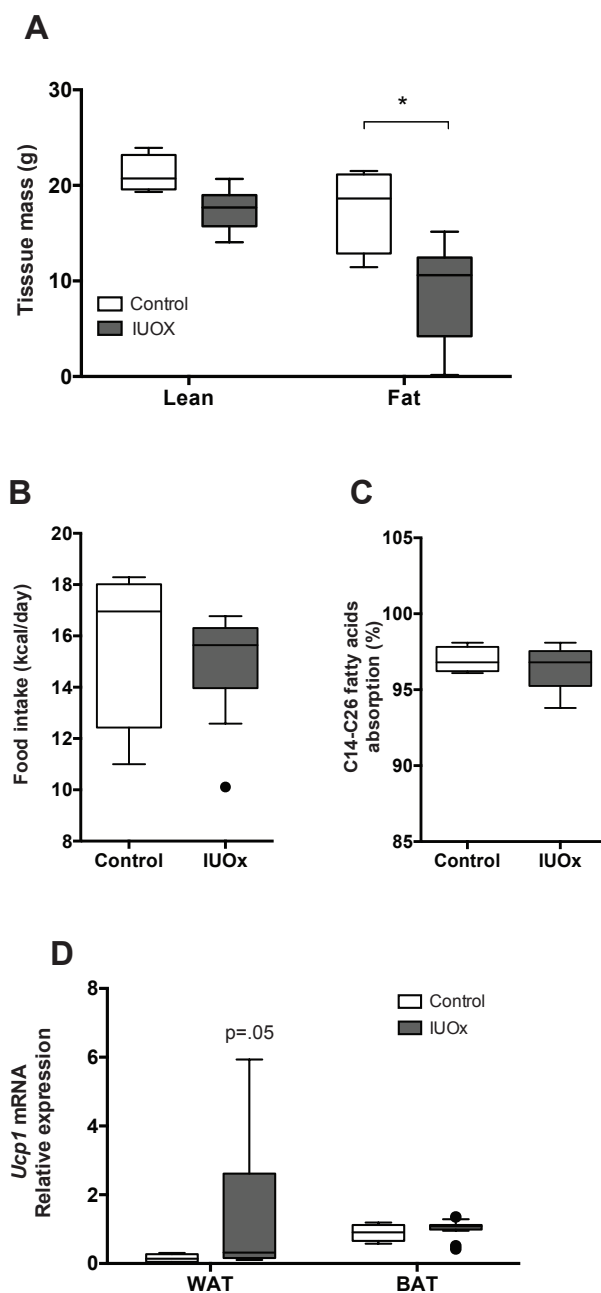


Figure 4: IUOX reduces body fat content by increasing energy dissipation via upregulation of Ucp1. A) Body composition analysis via pDEXA at 24 weeks of age, after 12 weeks of Western diet feeding. B) Food intake based on averaged values over 3 consecutive days (left) and corresponding long-chain fatty acid absorption (right). Mann-Whitney U-test. C) *Ucp1* mRNA expression levels in white and brown adipose tissue normalized to the expression of the ribosomal protein *36b4*. Data are presented as median and interquartile range (Tukey), n=4-13/group, Mann-Whitney U-test, * p<0.05.

cells, inducing DNA damage, lipid and protein oxidation, which are all associated with aging and cancer^{336,337}. However, during fetal development, a redox gradient is playing an essential role in tissue differentiation and vascularization⁶¹. A shift in this balance in the direction of increased oxidative stress would be expected to result in metabolic programming of the offspring with long-term negative effects increasing the risk for adult metabolic syndrome. Our data, however, revealed that in a model where increased oxidative stress is not accompanied by changes in fetal growth, ROS lead to a protective effect in the male offspring manifesting as resistance against diet-induced weight gain, reduced adiposity, and an overall improved metabolic phenotype. These results suggest that the perceived concept of oxidative stress leading to an increased susceptibility to adverse metabolic programming might require revision.

How can a supposedly adverse factor such as increased ROS confer protection against diet-induced metabolic dysfunction? In general, obesogenic diets and increased adiposity are associated with higher levels of systemic oxidative stress. Moderate levels of ROS, however, also fulfill an important physiological role in regulating gene expression and cell signaling pathways. Of note, relevant beneficial effects of exercise are largely conveyed by ROS; increased oxidative stress during exercise has been mechanistically shown to mitigate insulin resistance by altering the expression of ROS-sensitive transcriptional regulators of glucose homeostasis such as Ppar γ and Pgc1 α ⁶⁶. From these and similar observations the concept of “mitohormesis” developed⁶⁵. This theory suggests that exposure to small doses of a stressor such as ROS, can protect against larger subsequent doses, a process that engages a non-linear response to endogenously produced ROS upon cellular stress. In our model, an increased intrauterine ROS exposure conceivably conditions the metabolic network towards an improved response to an increasing oxidative and nutritional burden associated with the Western diet. Thus our data would be in agreement with the concept of mitohormesis extending it to the field of metabolic programming.

In our model, with equal energy intake, the IUOx group gained less weight, suggesting higher energy expenditure in these animals, which we, however, could not directly measure due to technical limitations. We therefore based our conclusions on the expression levels of Ucp1, which has been described as an adequate proxy for energy expenditure³³⁸. In general, Ucp1 expression is high in brown adipose tissue, where it mediates energy dissipation and heat generation. Available data suggest that Ucp1 could account for up to a quarter of the basal metabolic rate³³⁹. Consistent with a central role of Ucp1 in energy metabolism Ucp1 knockout mice were reported to be more susceptible to diet-induced obesity³⁴⁰, while transgenic animals are protected³⁴¹. White adipose tissue on the other hand usually serves for energy storage. However, independent of the role of Ucp1 in brown adipose tissue, it is also a key effector of the so-called “browning” of white adipose tissue, a condition, in

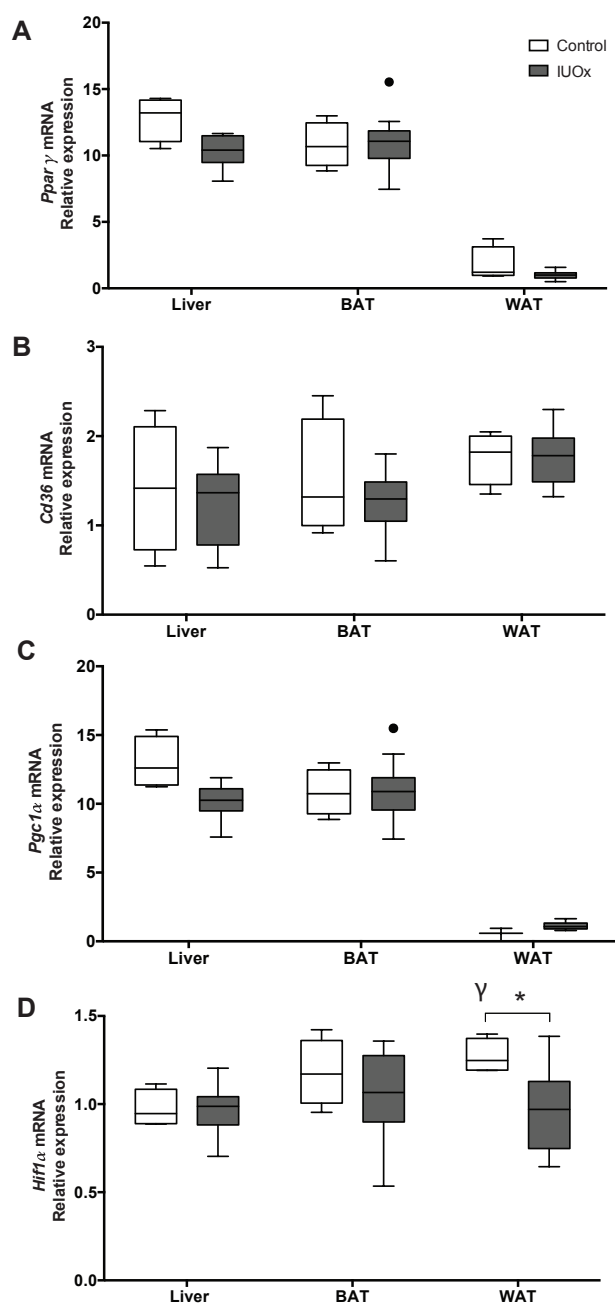


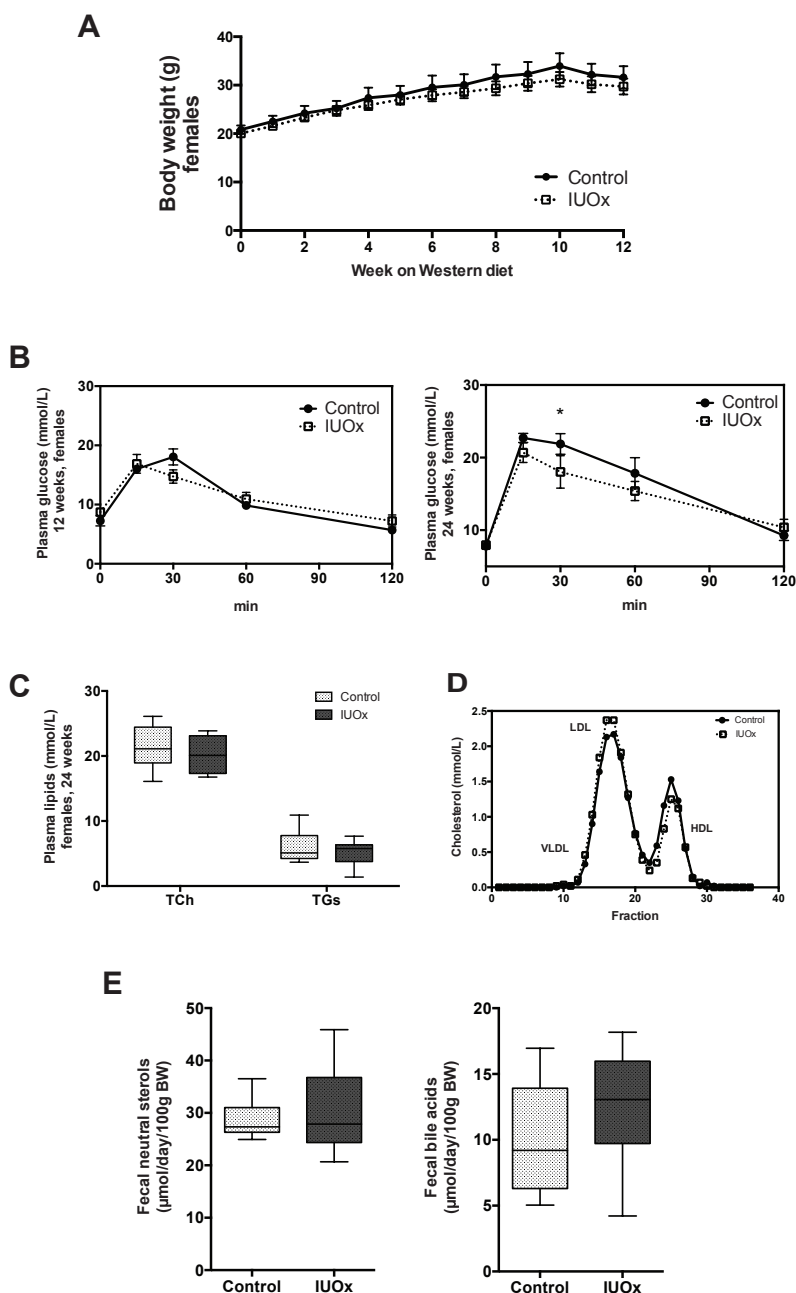
Figure 5: IUOx results in altered *Ucp1* expression in WAT by a *Ppar-γ* and *Pgc1α*- independent mechanism. A) qPCR-based gene expression of *Ppar-γ* and B) its model transcriptional target *Cd36* in liver, white (WAT) and brown adipose (BT) tissue after 12 weeks of Western diet. C) *Pgc1α*-expression levels and D) *Hif1α* expression. Expression levels are normalized to the respective expression of *36b4* in the corresponding tissue sample. Data are presented as median and interquartile range; n=4-13/group. Mann-Whitney U-test, * p<0.05.

which WAT participates in appreciable amounts in energy dissipation³⁴². This observation lends strong support to a causative contribution of the higher Ucp1 expression in WAT of IUOx mice observed in our study to the improved metabolic state in this group. The lack of differential expression of Ucp1 in female offspring, where a long-term metabolic impact of intrauterine oxidative stress was missing, also points towards a key role for Ucp1 in conveying the protective effects of IUOx.

Ucp1 was shown to be regulated by Nrf2³³⁴ as well as its transcriptional target Pgc1 α ³⁴³. However, in our experiment, the levels of Pgc1 α in white adipose tissue remained comparable, which indicates that likely a Pgc1 α -independent regulation of Ucp1 occurs in our model. Although this was not formally tested, we suggest that epigenetic regulation at the Ucp1 locus specifically in WAT offers a conceivable mechanistic explanation for the observed protection against an adverse diet-induced metabolic phenotype in the IUOx group in our study.

One relatively frequent finding of metabolic programming studies is the sex-specificity of the offspring's phenotype. Especially in the case of energy and lipid metabolism, the molecular and phenotypic outcomes of an adverse intrauterine environment are often more prominent in male offspring³⁴⁴⁻³⁴⁶. The sex of the embryo can modulate placental size, function and its ability to respond to adverse stimuli³⁴⁷, and hence sex-specific placental differences have been proposed to contribute to the sexual dimorphism in programming. However, the interaction of sex hormones and postpartum environment may also play a significant role in triggering programmed phenotypes³⁴⁸.

In summary, our data demonstrate that intrauterine exposure to oxidative stress results in resistance to diet-induced weight gain, adiposity, insulin resistance and dyslipidemia in adult mice. This response is restricted to the male offspring and is not observed in female littermates. Mechanistically we suggest a Pgc1 α -independent, likely epigenetic, upregulation of Ucp1 in white adipose tissue of IUOx mice with an associated increase in energy dissipation. These data might have important implications also for the interpretation of effects of oxidative stress during pregnancy on programming of metabolic disease in human populations.



Supplementary figure I: IUOx does not induce substantial metabolic changes in female mice after 12 weeks of Western diet challenge. A) Body weight change in female mice during 12 weeks of Western diet feeding. B) Glucose tolerance test before (left) and after (right) Western diet challenge. C) Total cholesterol and triglycerides in plasma in female mice. D) Cholesterol in lipoprotein subfractions after FPLC separation of pooled plasma ($n=5/\text{group}$) from female mice after 12 weeks of Western diet. E) Fecal excretion of neutral sterols (left) and bile acids (right). F) Relative expression (qPCR) of *Ucp1* (upper panel) and *Hif1 α* (lower panel) in female mice after

